

Proteomic and Mass Spectroscopic Quantitation of Protein S-Nitrosation Differentiates NO-Donors

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Cysteine residues in proteins play many important roles including in protein quaternary structure, cofactor binding, and as catalysts at enzyme active sites. Since the primary cysteine thiol is a good biological nucleophile and can attain multiple oxidation states, protein thiol residues represent natural targets for electrophiles and redox active agents including nitrosating and oxidizing species generated from NO. Cysteine modification is manifested in post-translational modification leading to modulation of protein function and regulation of biological processes (1, 2) and in triggering of cellular response to xenobiotics (3). S-Nitrosation, the chemical reaction of a thiol functional group with a nitrosonium equivalent, likely underlies the protein post-translational modification commonly referred to as protein S-nitrosylation. Such protein nitrosylation has been argued to rival phosphorylation in importance as a cell signaling process (4).

A large number of protein thiols have been reported to undergo regulatory nitrosation, for example, S-nitrosation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is proposed to facilitate nuclear translocation and apoptosis (5). It has even been proposed that many, if not most, biological effects of NO are mediated by protein S-nitrosation (6). The proclivity of the active site thiol in cysteine proteases toward nitrosothiol formation is considered as providing a mechanism of negative regulation of function by endogenous NO and exogenous NO-donors (7), and NO production may itself be regulated by nitrosation of the Zn-tetrathiolate structure of nitric oxide synthase 2 (iNOS) (8). Glutathione S-transferase P1-1 (GST-P1), found at elevated levels in many solid and chemoresistant tumors (9), is a further

ABSTRACT Protein S-nitrosation has been argued to be the most important signaling pathway mediating the bioactivity of NO. This post-translational modification of protein thiols is the result of chemical nitrosation of cysteine residues. The term NO-donors covers very different chemical classes, from clinical therapeutics to probes of routine use in chemical biology; their different chemistry is predicted to result in distinctive biology regulated by protein S-nitrosation. To measure the extent of protein S-nitrosation by NO-donors, a proteomic mass spectrometry method was developed, which quantitates free thiol *versus* nitrosothiol for each modified cysteine residue, coined d-Switch. This method is adapted from the biotin switch (BST) method, used extensively to identify S-nitrosated proteins in complex biological mixtures; however, BST does not quantitate free thiol. Since glutathione-S-transferase P1-1 (GST-P1) has been proposed to be a biological “NO-carrier”, GST-P1 was used as a reporter protein. The 5 different chemical classes of NO-donors compared by d-Switch demonstrated very different profiles of protein S-nitrosation and response to O₂ and cysteine, although all NO-donors were oxidants toward GST-P1. The low limits of detection and the ability to use established MS database searching allowed facile generalization of the d-Switch method. Therefore after incubation of neuronal cell cultures with nitrosothiol, it was possible to quantitate not only S-nitrosation of GST-P1 but also many other proteins, including novel targets such as ubiquitin carboxyl-terminal esterase L1 (UCHL1). Moreover, d-Switch also allowed identification of non-nitrosated proteins and quantitation of degree of nitrosation for individual protein thiols.

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protein reported to be nitrosated, in this case at the most reactive cysteine (Cys47) and proposed to act as a biological “NO-carrier” (10–12). GST-P1 was therefore a reasonable target to compare and to quantitate protein *S*-nitrosation in response to 5 different classes of NO-donors.

The term “NO-donor” is widely used in the literature for (i) diazeniumdiolates that are true NO-donors, releasing predictable fluxes of NO; (ii) the endogenous nitrosothiols, *S*-nitrosocysteine (CysNO) and *S*-nitrosoglutathione (GSNO) that function as NO⁺ donors; (iii) organic nitrites that are in clinical use and are efficient NO⁺ donors; (iv) clinical organic nitrates that release NO₂ and require reductive bioactivation to give NO; and (v) oxadiazole-*N*-oxides that are in preclinical studies and again require bioactivation, possibly to NO and HNO. It would be more accurate to term these compounds NO-mimetics, since they result in NO bioactivity, including *via* protein *S*-nitrosation, which is not derived solely from donation of nitrogen monoxide (NO). The clinical importance of these collective “NO-donors” and their different chemistries demands a quantitative comparison of their ability to effect protein *S*-nitrosation; toward this end a novel adaptation of the biotin switch (BST) method was developed, coined d-Switch.

The BST methodology introduced by Jaffrey *et al.* has been central to reports of protein *S*-nitrosation (13). This semiquantitative method consists of three principal steps: blocking of free thiols with a thiol reactive reagent, reducing nitrosothiol to free thiol with ascorbate, and labeling the resulting free thiol with biotin (Figure 1). A number of sources of artifacts have been identified in the BST approach, resulting in publication of a number of modifications and improvements; most recently, the SNO-RAC methodology was reported, which incorporates iTRAQ quantitative proteomic techniques (Figure 1) (14). In concert with LC-MS/MS chromatography, both the BST and SNO-RAC methodologies have the potential to identify nitrosated proteins, the number of which detected may run to the hundreds in cell culture studies. The question remains, however, as to whether the extent of cysteine nitrosation in these proteins is sufficiently high to lead to altered protein function. The objective of the present research was to answer the following question: Can we quantitate the percentage of a critical protein thiol that is nitrosated by different NO-donor classes? The d-Switch method developed to answer this question is complementary to BST, does not

use proprietary reagents, and is generalizable in cell culture. Protein *S*-nitrosation measured by BST and d-Switch was compared for five classes of NO-donors in purified protein and cell cultures, demonstrating the ability of d-Switch to quantify nitrosation and revealing the quite different protein *S*-nitrosation properties of NO-donors, routinely used in chemical biology and drug discovery.

RESULTS AND DISCUSSION

There is no doubt that under certain cellular conditions, selected cysteine residues in proteins undergo nitrosation. There is also convincing evidence that *S*-nitrosation can influence protein function, most simplistically in enzyme inhibition by nitrosation and blocking of catalytic, active site cysteine residues such as in caspases and protein phosphatases (7, 15). In such cases, the percentage of non-nitrosated, free cysteine thiol will directly correlate with enzyme activity and hence biological function. Evidence has been provided that protein thiol nitrosation mediates cell signaling by NO in parallel with signaling *via* NO/cGMP. Termed protein *S*-nitrosylation, this signaling mechanism is proposed to represent a broad-based post-translational regulation of protein function (16). Much of the evidence provided for *S*-nitrosation employs BST immunoassay analysis, followed by elegant studies mutating the relevant cysteine, leading to loss of biological response accompanied by loss of protein *S*-nitrosation visualized by BST. One example of the use of BST in this way is *S*-nitrosation of the primary cellular target of NO, soluble guanylyl cyclase (sGC), which has been proposed to be regulated through *S*-nitrosation by the “NO-donors”, CysNO, GSNO, and nitroglycerin (GTN) (17, 18). This work led to the proposal that inhibition of sGC through *S*-nitrosation by GTN was the cause of clinical nitrate tolerance (17). This work has been queried recently (19), demonstrating the importance of both a quantitative technique for measuring *S*-nitrosation and a fuller understanding of agents commonly referred to as NO-donors. A simple method that quantified amounts of protein Cys-SNO relative to the non-nitrosated free protein thiol (Cys-SH) would be invaluable to support a functional role for protein *S*-nitrosation.

The term NO-donors has come to include newer compounds designed to deliver defined fluxes of NO under specific conditions, in addition to nitrite and nitrate nitrovasodilators that have been in clinical use for well

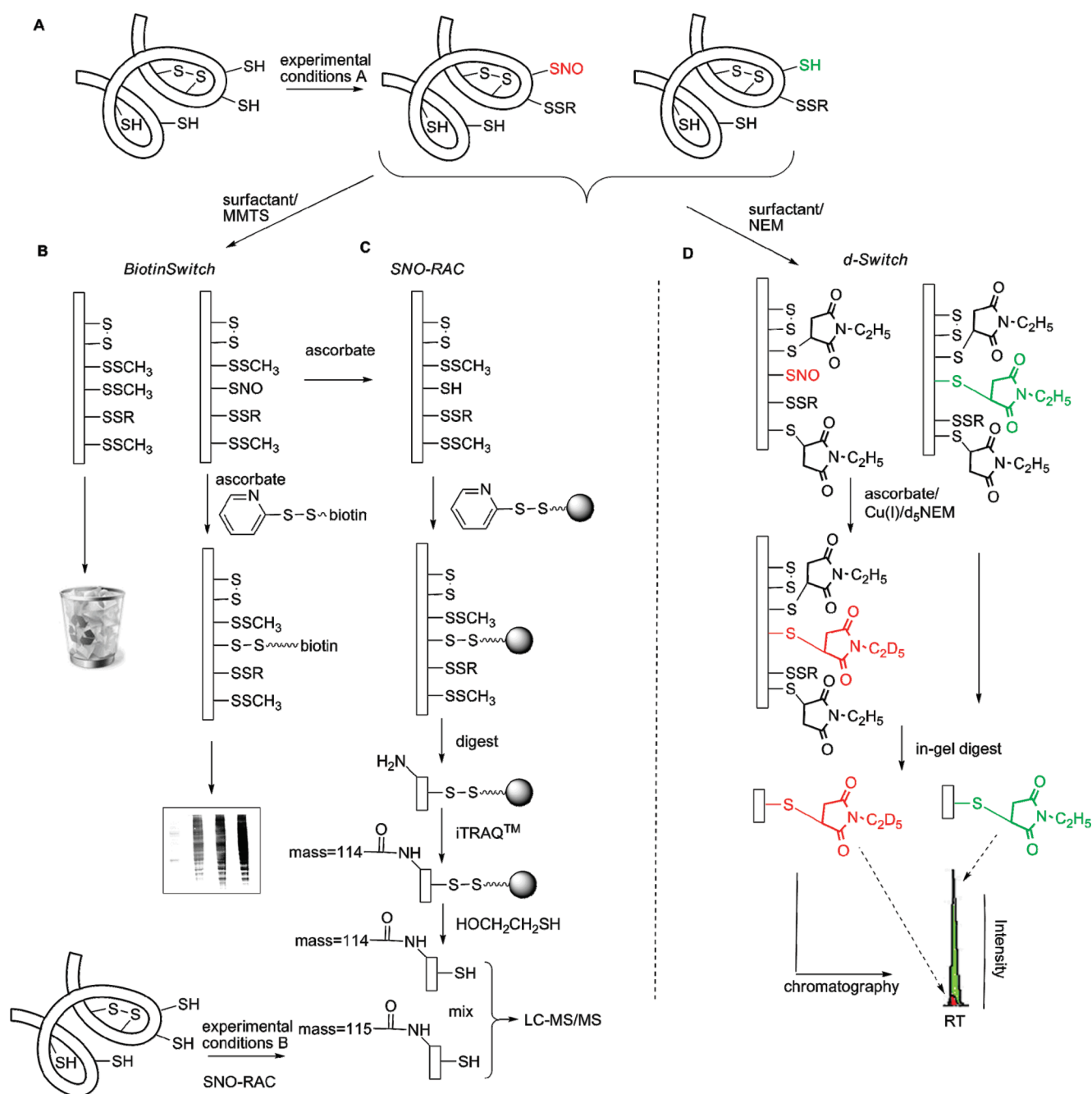


Figure 1. Schematics for biotin switch (BST), SNO-RAC, and d-Switch methods. **A**) The proteome may contain cysteines with varied reactivity and accessibility and intramolecular disulfide bonds; BST and modified BST methods discard non-nitrosated proteins. **B**) The standard BST method visualizes biotinylated proteins by Western blot (shown) or may identify proteins by LC-MS/MS chromatography after pull-down of biotinylated proteins prior to protein digestion. **C**) The SNO-RAC is a modified BST methodology that uses activated disulfide beads in place of biotin to isolate nitrosated proteins in combination with iTRAQ quantitative proteomic analysis. **D**) The d-Switch modification is designed to quantify the absolute ratio of nitrosated *versus* non-nitrosated, reduced cysteine at individual sites in key proteins; therefore, non-nitrosated proteins must be isolated and quantified, not discarded.

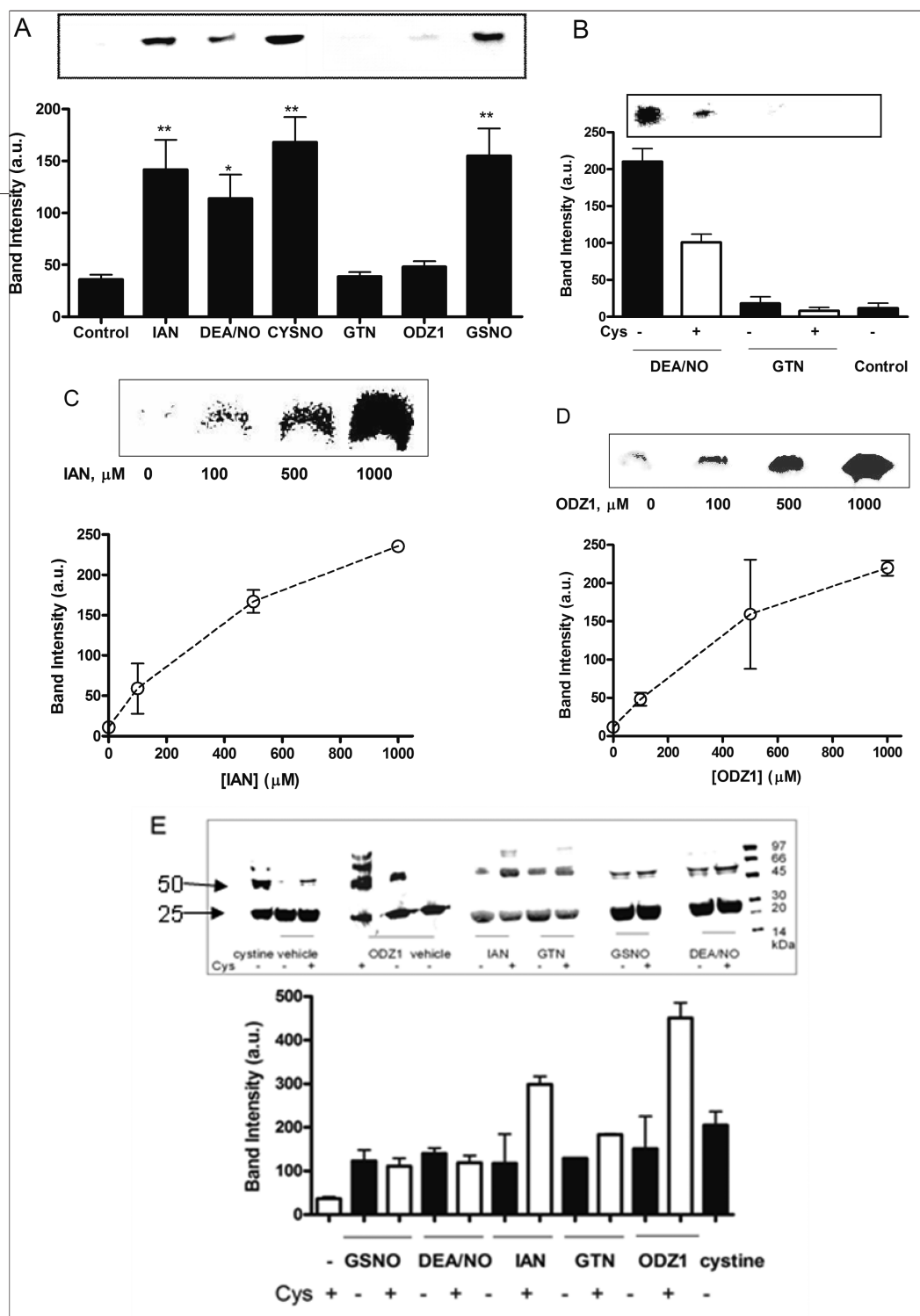


Figure 2. Comparison of GST-P1 nitrosation and oxidation by NO-donors. **A)** BST analysis of GST-P1 (20 μ M) treated with NO-donors (500 μ M) at 37 $^{\circ}$ C for 30 min. ANOVA analysis with Dunnett's post test relative to vehicle control: ** $p < 0.001$; * $p < 0.005$. Response to IAN, GSNO, CysNO, and DEA/NO was not significantly different. **B)** BST analysis of GST-P1 (20 μ M) untreated (control) or incubated with NO-donor (500 μ M) in the presence and absence of Cys (2 mM). Quantitation was by image densitometry from blots. **C)** BST analysis of GST-P1 (20 μ M) nitrosation after incubation with varying concentrations of IAN at 37 $^{\circ}$ C for 30 min. **D)** BST analysis of GST-P1 (20 μ M) nitrosation after incubation with varying concentrations of ODZ1. **E)** Nonreducing SDS-PAGE analysis of GST-P1 oxidation to dimer (and higher oligomers) by NO-donors (500 μ M) visualized by Coomassie blue staining. GST-P1 (20 μ M) was incubated with NO-donors (500 μ M) or cystine (1 mM) in the presence and absence of Cys (2 mM) at 37 $^{\circ}$ C for 30 min. Oxidation of GST-P1 was significantly increased in the presence of cysteine for IAN, GTN, and ODZ1, but not for other NO-donors. Quantitation was by image densitometry from blots; upper panels show representative blots. Data show mean and SD in arbitrary units for triplicate experiments, which for oxidation/dimerization were normalized to the untreated control as 0%.

over a century (20–23). There is evidence in the literature that the very different chemistry of these NO-donors inevitably delivers differing biological responses; however, in many reports NO-donor classes are treated as equivalent. Selected for this study were the endogenous low molecular weight nitrosothiols, GSNO and CysNO; the clinical nitrite vasodilator, isoamyl nitrite (IAN); the clinical nitrate, GTN; and a novel oxadiazole-*N*-oxide (furoxan), ODZ1. The latter is a drug class that has been the subject of recent intensive studies in NO-donor drug discovery (24, 25). The fast release diazeniumdiolate (DEA/NO) was also selected for study because of the extensive use of diazeniumdiolates in chemical biology.

Human GST-P1 was chosen as a target, in part because of multiple reports of its *S*-nitrosation. GST-P1, the most widely distributed of the GST phase 2 detoxifying enzymes (26, 27), is found at elevated levels in solid tumors (e.g., breast, lung colon, pancreas) and in drug-resistant tumors (9) and both detoxifies and is adducted by electrophilic xenobiotics (28, 29). The 4 cysteine residues in GST-P1 are modified with reactivity Cys47 > Cys101 > Cys14 >> Cys169; although not part of the active site, modification does not necessarily lead to loss of enzyme activity. In 2001, Lo Bello *et al.* using ESI-MS analysis reported that in the absence of GSH, GST-P1 was nitrosated by the endogenous nitrosothiol, GSNO, and proposed GST-P1 as a cellular NO-carrier (10). Evolution of this hypothesis viewed GST-P1 as an important endogenous carrier of a dinitrosyl-iron complex (11, 30, 31); however, *S*-nitrosation of Cys47 by GSNO was still claimed to be a likely physiological scenario (11, 12).

GST-P1 Nitrosation by NO-Donors. Previous reports on GST-P1 nitrosation using 2 mM GSNO estimated a half-life of 5 min for Cys47 nitrosation (10, 11). Although the concentration of GSNO was supraphysiological, the studies confirmed *S*-nitrosation. Organic nitrites, such as IAN, are excellent nitrosating agents; the observed rate of reaction with glutathione (GSH; $k \approx 2 \text{ M}^{-1} \text{ s}^{-1}$) indicates that, as for the nitrosothiols, extensive nitrosation of GST-P1 by IAN should be anticipated (32). GST-P1 nitrosation was assayed by BST for all six NO-donors (500 μM) demonstrating qualitatively that the diazeniumdiolate, nitrite, and nitrosothiols were effective nitrosating agents, whereas GTN and ODZ1 were at best weak nitrosating agents. The increase in GST-P1 nitrosation by DEA/NO, GSNO, CysNO, and IAN was significant, but BST was unable to show any significant

differences between these four NO-donors (Figure 2, panel A).

Both nitrates and furoxans may require reductive bioactivation to function as NO-donors; although likely mediated by multiple enzymes, bioactivation mediated by free thiol has frequently been proposed (21, 23, 25, 33). Addition of cysteine (2 mM) to incubations of GST-P1 with GTN did not cause *S*-nitrosation, whereas cysteine significantly reduced GST-P1 nitrosation by the spontaneous NO-donor DEA/NO (Figure 2, panel B). The observed loss of signal for nitrosated protein is compatible with accelerated denitrosation by the added cysteine (34); moreover, cysteine can compete with GST-P1 for nitrosating species generated from NO autoxidation. The next step was to use BST to examine concentration-dependent protein *S*-nitrosation, as frequently employed in the literature. IAN, which gave significant nitrosation at 500 μM , and the marginal nitrosating agent ODZ1 were studied, the resulting blots apparently showing that both NO-donors were able to elicit protein *S*-nitrosation (Figure 2, panels C and D). This experiment illustrates a potential pitfall with BST, because as we shall see from d-Switch analysis, GST-P1 nitrosation by ODZ1 is real but low: $1.2 \pm 0.3\%$ nitrosation of Cys47.

GST-P1 Oxidation by NO-Donors. GST-P1 is readily oxidized to form oligomers with intermolecular and intramolecular disulfide bonds (27) and thiols are known to undergo oxidation to disulfides in nitrosating solutions and on reaction with GTN (35–37). Oxidative dimerization of GST-P1 was observed for all NO-donors studied by nonreducing SDS-PAGE: GTN was a potent oxidant, and oxidation was dependent on NO-donor concentration (Supplementary Figure 1). Again significant differences were observed between the chemical classes in their response to added cysteine: oxidation was not significantly enhanced for GSNO or DEA/NO but was enhanced by cysteine (2 mM) for ODZ1 > IAN > GTN (Figure 2, panel E). Cystine (1 mM) was also observed to enhance GST-P1 oxidative dimerization and likely contributed to the enhanced oxidation observed when cysteine is added to IAN, GTN, and ODZ1. Cu/ascorbate treatment used to convert RS-NO to R-SH in BST and d-Switch methods did not reduce protein disulfide bonds, since allowing GST-P1 to dimerize followed by treatment with Cu/ascorbate was without effect (Supplementary Figure 6).

GST-P1 Nitrosation by d-Switch. The d-Switch method depicted in Figure 1 is a proteomics method adapted from the BST assay, in which NEM is used in place of MMTS for Cys-SH blocking, and d_5 -NEM is used in place of biotin-HPDP for Cys-SNO labeling (Figure 1). The use of CuCl/ascorbate was chosen for the denitrosation/labeling step, because of the enhanced signal (Supplementary Figure 2) (38). The d-Switch method relies upon protein digestion to yield a peptide containing the protein-Cys of interest that is amenable to analysis by LC-MS/MS; in studies on GST-P1, this is Cys47. In-gel tryptic digest of GST-P1 monomer followed by LC-MS/MS analysis revealed the desired decapeptide at 12.8–13.4 min in the total ion current chromatogram, the identity of which was confirmed by MS/MS analysis of the doubly charged peptide (Supplementary Figure 2). Reaction times and temperatures and reagent concentrations were optimized at each step in the d-Switch methodology yielding an assay in which the NEM and d_5 -NEM labeled peptides were readily quantified in extracted ion chromatograms (m/z 603.3 (d_0) versus 605.6 (d_5) for $z = 2$; Figure 3, panel A). Both NEM and d_5 -NEM labeled peptides have almost identical retention time and identical ionization efficiency, and hence calculating the peak area ratios of the two peptides from the extracted ion chromatogram allows for quantitative analysis of Cys47 nitrosation. The final output of d-Switch analysis is the peak area ratio of Cys-SNO labeling (d_5) to the summed Cys-SNO plus Cys-SH labeling ($d_0 + d_5$), reported as percentage nitrosation: $\%[d_5/(d_0 + d_5)]$.

The precision of the d-Switch analysis compared to BST was demonstrated in the measurement of concentration- and time-dependent S-nitrosation of GST-P1 by IAN (Figure 3, panels B and C). Measured at 37 °C and 30 min, percent nitrosation of Cys47 was linear in IAN concentration; however, at 10 μ M IAN, Cys47 was only $0.43 \pm 0.04\%$ nitrosated. The rate constant for hydrolysis of IAN has been measured spectrophotometrically in aqueous phosphate buffer at 37 °C to be $1.18 \times 10^{-3} \text{ s}^{-1}$, corresponding to a half-life of approximately 8 min and a second order rate constant for reaction with water of $2 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$ (39, 40). The rate constant for the nitrosation of cysteine by IAN in phosphate buffer (pH 7.0 and 25 °C) has been reported ($k = 1.05 \text{ M}^{-1} \text{ s}^{-1}$), as has the rate constant for the fast reac-

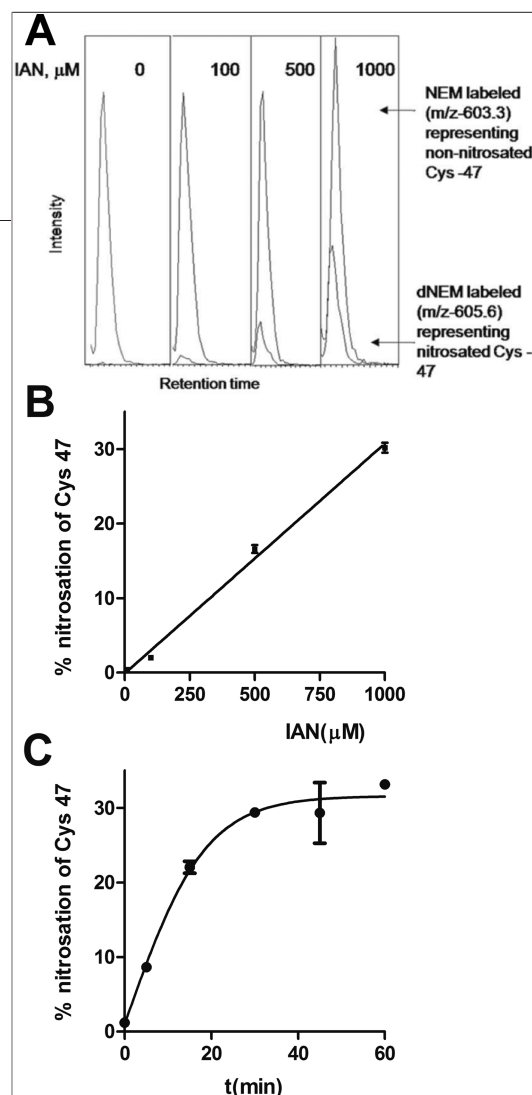


Figure 3. Concentration- and time-dependent nitrosation of GST-P1 by IAN quantified by dSwitch. GST-P1 (20 μ M) was treated with IAN at various concentrations, and the reaction was quenched at various time points by addition of NEM. **A)** Representative extracted ion chromatograms showing increasing intensity of d_5 -NEM labeled peptide ASC[d_5 -NEM]LYGQLPK $^{2+}$ ($m/z = 605.6$) derived from GST-P1 Cys47 nitrosation versus NEM labeled peptide ASC[NEM]LYGQLPK $^{2+}$ ($m/z = 603.3$) derived from Cys47 free thiol after 30 min of treatment with IAN (0, 0.1, 0.5, 1 mM). The retention time of each of the four chromatogram excerpts is 12.6–13.6 min. The ratio of peak areas was used to calculate percent nitrosation: $A_{\text{Cys47-SNO}} / (A_{\text{Cys47-SNO}} + A_{\text{Cys47-SH}})$. **B)** Percentage nitrosation of GST-P1 Cys47 was dependent on NO-donor concentration. Data show mean and SEM of triplicate experiments. **C)** Percentage nitrosation of GST-P1 Cys47 by IAN (1 mM) was dependent on incubation time as calculated from extracted ion chromatogram peak areas. Data show mean and SEM of triplicate experiments.

tion with GSH in an aqueous biological compartment ($k \approx 2 \text{ M}^{-1} \text{ s}^{-1}$) (41). The observed time course for trapping of IAN by Cys47 to give protein nitrosothiol was therefore surprisingly slow (Figure 3, panel C), suggesting that reactions in addition to hydrolysis and transni-

nitrosation determine the percent nitrosation observed. For example, intermolecular disulfide formation, observed to be induced by all NO-donors (Supplementary Figure 1), consequent to Cys47 nitrosation, is one such secondary reaction that leads to loss of Cys47-SNO.

Comparison of NO-Donors Using d-Switch and Effect of O₂. The concentration dependence of Cys47 nitrosation was measured for six NO-donors (Figure 4, panel A). The two nitrosothiols, CysNO and GSNO, were the most efficient nitrosating agents, outperforming the true NO-donor DEA/NO that theoretically yields twice the amount of NO. The nitrite, an efficient and direct nitrosating agent, was in fact less efficient than the thionitrites (nitrosothiols), because of competing hydrolysis. At lower concentrations of CysNO and GSNO (10 μ M), less than 5% nitrosation of GST-P1 was observed. Under high physiological fluxes of NO, the estimated [GSNO] is 1–5 μ M (42), and therefore the prediction from extrapolation of these data is that significant S-nitrosation of GST-P1 is unlikely to be observed under physiological conditions. Cys47 nitrosation by GTN was not observed and the levels of nitrosation induced by ODZ1 were only significantly above background at millimolar concentration. The d-Switch technique combines sensitivity with precision, as evidenced by the low variance and the ability to discriminate quantitatively between NO-donors in Cys47 nitrosation, which was not possible using BST analysis. S-Nitrosation by NO is expected to be dependent on O₂, since oxidation of NO to nitrosating N₂O₃ is the dominant aerobic mechanism (43). Assuming that diazeniumdiolates are the only true NO-donors of the chemical classes studied herein, the prediction was that Cys47 nitrosation by DEA/NO would require O₂, which was borne out by observations in 20% versus 1% O₂ where nitrosation was reduced from 11% to 1.8% (Figure 4, panel B). All other NO-donor classes showed no dependence on O₂, demonstrating that Cys47 nitrosation is not mediated *via* release of NO from these agents (Scheme 1).

S-Nitrosation induced by NO-donors from five different chemical classes was studied using GST-P1 as a model protein, measuring the extent of nitrosation of Cys47, and examining the influence of O₂ and added cysteine. Only 3 of the 5 drug classes gave significant S-nitrosation. Of these 3, only the true NO-donor diazeniumdiolate required aerobic conditions to effect nitrosation *via* generation of N₂O₃. In contrast, all NO-donors

oxidized GST-P1 to give dimeric protein disulfides. The combined analysis demonstrated that the different chemistry of NO-donor classes is reflected in differing profiles of protein nitrosation and oxidation. Since oxidation of protein thiols to disulfides by NO-donors is concomitant or dominant over nitrosation, cysteine oxidation may be responsible for many actions of endogenous NO and NO-donors. Theoretical and experimental studies have reported thiol oxidation, rather than nitrosation, as the dominant modification induced by NO (44, 45), although this is often overlooked when considering mechanisms of NO bioactivity. A variety of oxidative modifications of protein thiols have been reported for NO and NO-donors, including disulfides and sulfenic and sulfinic acids (46–48).

Transnitrosation of GST-P1-Cys47 by nitrosothiols was observed not to reach completion. The d-Switch analysis indicated that at millimolar nitrosothiol, significant amounts of free thiol persisted; the ratio of Cys47SH to Cys47SH + Cys47SNO was 45%. The extent of protein nitrosation is expected to be determined by the balance of nitrosation *versus* denitrosation rates; the apparent saturation of Cys47 nitrosation by nitrosothiols at 55% might be in accord with this equilibrium in the transnitrosation reaction between small molecule and protein thiol (Scheme 1). Another possibility was that Cys47 was “hidden” from small molecule nitrosothiol in the interface of GST-P1 homodimers, since this protein is known to dimerize noncovalently. To test these hypotheses, an experiment was performed incubating GST-P1 at 10-fold lower concentration with CysNO; however, the degree of Cys47 nitrosation was not increased (Supplementary Figure 12). Mitchell *et al.* reported on S-nitrosation of caspase-3, a protein reported to be basally nitrosated in nature, using GSNO and a peptide-SNO complementary to the active site and rigorous use of BST and mass spectrometry (49). Comparison of comparable GSNO and peptide-SNO esters (0.5 mM) in cell culture showed almost 3-fold less S-nitrosation of caspase-3 by GSNO. Furthermore, GSNO treatment of pure protein gave protein-disulfide formation. For GST-P1, S-nitrosation did not increase above 0.5 mM CysNO (Figure 4, panel A), whereas SDS-PAGE assay of the concentration dependence of GST-P1 oxidative dimerization showed that oxidation increased 3-fold with increasing [CysNO], from 0.5 to 2 mM (Supplementary Figure 13). The importance of thiol oxidation as a

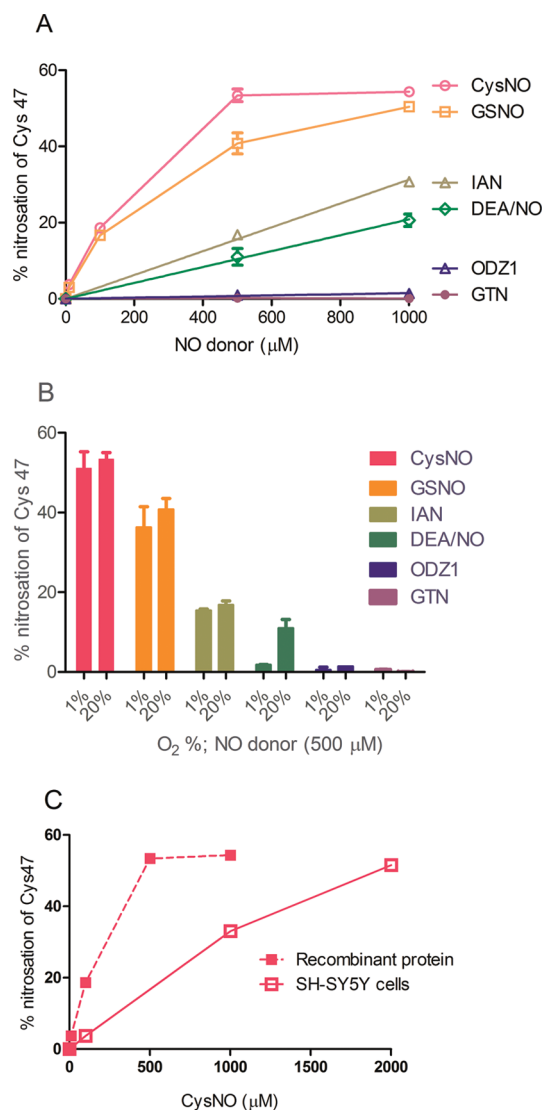


Figure 4. d-Switch comparison of GST-P1 nitrosation by six NO-donors in pure form and in human cells. A) Concentration-dependent Cys47 nitrosation after incubation of GST-P1 (20 μM) with the indicated concentrations of various NO-donors at 37 °C for 30 min from d-Switch analysis. Data showing mean and SEM of 3 experiments for IAN, DEA/NO, and ODZ1 are linearly fit with slopes of 0.031, 0.021, and 0.0014, respectively. B) Effect of O₂ pressure on GST-P1 nitrosation at Cys47 by NO-donors (500 μM) analyzed by d-Switch. Data show mean and SD of 3 experiments. C) Concentration-dependent Cys47 nitrosation after incubation of human neuroblastoma SH-SY5Y cells with CysNO for 20 min and analysis by d-Switch (solid line; open square); data for recombinant protein incubation with CysNO are shown for comparison (dashed line; solid square).

competing and consequent reaction to nitrosation is apparent from these data. Further mechanistic analysis clearly requires parallel measurement of disulfide formation. Indeed, this was the primary impetus for development of an alternative methodology to BST, which unlike BST is not dependent on formation of disulfides in the analytical steps. Adaptation of d-Switch using tris(2-carboxyethyl)phosphine in place of Cu/ascorbate is currently under study.

GST-P1 Nitrosation in Cells. Evidence for GST-P1 as an NO-carrier was reported in *E. coli* cells used to over-express GST-P1, by exposing cells to 2 mM GSNO or 0.5 mM DEA/NO (11). Herein, under similar conditions, GST-P1 nitrosation was quantified by d-Switch in living cells. *E. coli* cells overexpressing GST-P1 were exposed to NO-donor; however, very low levels of nitrosation were observed, and in order to measure 8% Cys47 nitrosation, combinations of bolus injections of DEA/NO were required to give total delivery of 40 mM NO (Supplementary Figure 3C). Unsurprisingly, the same experiment performed with GTN as NO-donor yielded no signal corresponding to nitrosation. More interesting was the measurement of protein nitrosation in human neuronal cells.

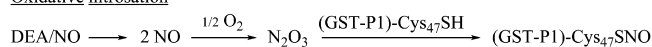
SH-SY5Y neuroblastoma cells were treated with different concentration of CysNO (0.1, 1, 2 mM) and divided into two sets: (i) for BST analysis and (ii) for d-Switch analysis. BST analysis showed the expected concentration dependent increases in multiple S-nitroso-proteins (Supplementary Figure 8). After d-Switch treatment, the gel region corresponding to GST-P1 was subjected to in-gel tryptic digestion and analyzed by nLC-LTQ-FT-ICR. The resulting MS and MS/MS data were searched using MassMatrix against the IPI human v3.65 database (50). GST-P1 was identified with excellent sequence coverage of 66%. The MS spectrum for ASCLYGQLPK, the GST-P1 peptide containing Cys47, was searched using the scan number generated from the database search and comparing with the observed full scan MS spectrum. The MS spectrum shown in Figure 5, panel A, showing unlabeled (Cys47-SH; green) and d₅-labeled (Cys47-SNO; red) peptide after CysNO treatment of cells, vividly displays the capability of the d-Switch methodology. The GST-P1 peptide fragment, labeled at Cys47 with NEM and/or d₅-NEM was rigorously identified using MS/MS (Supplementary Figure 7A). The concentration-dependent nitrosation of GST-P1 Cys47

SCHEME 1. Reaction schemes for GST-P1 S-nitrosation by CysNO, IAN, and DEA/NO, showing oxygen dependence for NO, and for GST-P1 oxidation by GTN (RONO₂) and subsequent to nitrosation

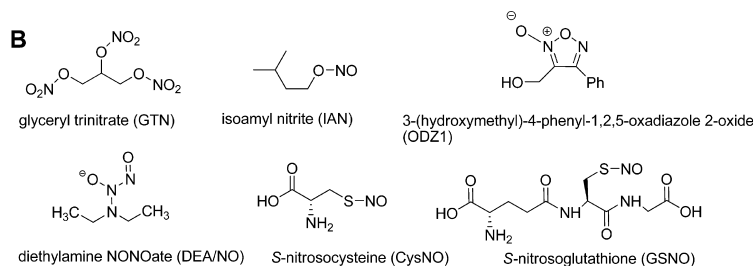
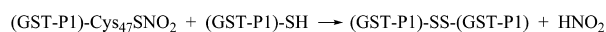
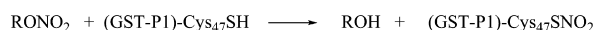
A Transnitrosation



Oxidative nitrosation



Oxidation



was quantified using the peak height of the most intense isotope peak in the *d*₅-NEM-peptide compared to the corresponding peak in the NEM-peptide. Concentration-dependent nitrosation of GST-P1 was again observed by CysNO (Figure 4, panel C, solid line; open square). At 2 mM CysNO, the extent of GST-P1 S-nitrosation in cells was the same as that observed in recombinant protein at 1 mM CysNO, whereas at 100 μM CysNO, Cys47 nitrosation was 5-fold lower in cell culture. The extent of GST-P1 Cys47 nitrosation was less than 5% at 100 μM CysNO. In comparable experiments, treatment of SH-SY5Y cells with 5 mM DEA/NO resulted in less than 5% nitrosation of GST-P1 at Cys47, and treatment with GTN gave no nitrosation. BST analysis of cell cultures incubated for up to 24 h with GTN (1 mM) showed no time-dependent increase in signal and no significant differences with untreated control blots (Supplementary Figure 10).

The efficient nitrosothiol nitrosating agents at close to physiological concentrations (10 μM) gave only 3.4 ± 0.5% Cys47 nitrosation, and nitrosation was never

observed to exceed 55% even in the presence of nitrosothiols at millimolar concentrations. These observations are not compatible with a proposed physiological role as a NO-carrier but certainly do not rule out the proposal of GST-P1 as a carrier of dinitrosyl-Fe complex (10–12, 30, 31). Previous application of SNO-RAC and SNO-CAP methods indicated two pools of S-nitrosated proteins, GSH stable and GSH labile, the latter defined by rapid denitrosation by GSH (14, 51). Of 102 S-nitrosated proteins detected in rat brain homogenates by SNOCAP after incubation with GSNO (40 μM) for 20 min, 10 were in the stable pool; however, GST-P1 Cys47 nitrosation was not detected.

Human GST-P1 has been assigned key roles in response to

NO and oxidative stress (10–12, 30, 31). Cytoprotective roles include inhibition of c-Jun N-terminal kinase (JNK) mediated apoptosis (52) and coordinated regulation of stress kinases in response to oxidative stress (53). A role for GST-P1 has recently been proposed in mediating glutathionylation following oxidative and nitrosative stress, induced by exogenous NO or disulfide (54), wherein GST-P1 was reported to be glutathionylated at Cys47 and Cys101, impacting the binding of JNK. NO and ROS induced disulfide formation at Cys47 is also likely to play an important role in regulation of peroxiredoxins through formation of heterodimers (55, 56). The observation that all NO-donors induced Cys47 oxidation to give GST-P1 homodimers confirms that GST-P1 may respond to nitrosative stress by protein and glutathione disulfide formation.

Cellular Protein Nitrosation beyond GST-P1. The original BST technique has frequently been modified, including the addition of LC-MS to identify S-nitrosated proteins. The SNOSID (SNO site identification) technique was introduced to identify S-nitrosated proteins

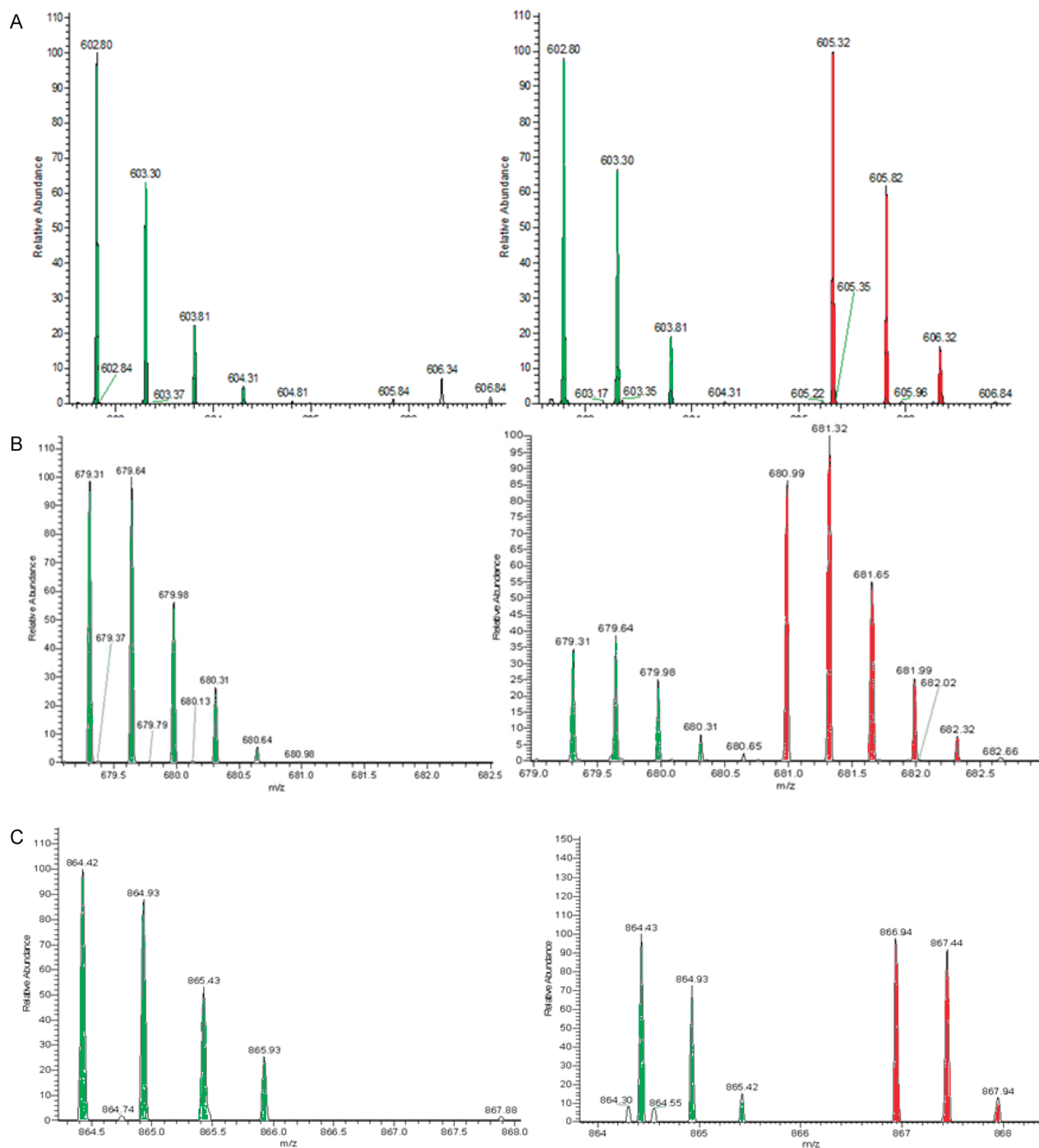


Figure 5. Nitrosation of proteins in SH-SY5Y cells analyzed by LC-MS/MS. LTQ-FTMS spectra of NEM and d_5 -NEM labeled peptides from d-Switch analysis of CysNO-treated SH-SY5Y cells. On the left, the spectrum is of the control experiment without CysNO. On the right, treatment of intact cells with CysNO (2 mM) gave spectra showing d_0 -NEM and d_5 -NEM labeled peptides. A) Cys47 peptide of GST-P1; the green signals are natural isotope distribution for the peptide corresponding to the non-nitrosated peptide, and the red peaks are the similar isotope signals corresponding to peptide from GST-P1 nitrosated at Cys47. As displayed in Figure 4, panel C, under these conditions GST-P1 Cys47 is 51% nitrosated. B) Cys 152 peptides of UCHL1. C) Cys53 peptides of DJ-1.

in complex biological mixtures using LC-MS after tryptic digest of BST treated proteins followed by avidin capture of biotinylated peptides (57). In another study His-tag idoacetamide was used for affinity capture of peptides obtained by trypsinolysis (58). Other laboratories have reported combinations of BST with 1D and 2 D PAGE and in-gel trypsinolysis to identify nitrosated proteins by LC-MS (59). The SNOCAP (*S*-nitrosothiol capture) modified BST procedure introduced in 2008 bears some similarity to d-Switch in that quantitation is achieved by heavy and light isotope labeling but uses biotinylated disulfide reagents (51). Recently, Stamler reported the SNO-RAC methodology, a modification of the BST method, which incorporates iTRAQ quantitative proteomic techniques to map the “cysteome” (Figure 1) (14). From our perspective, there was a need for a technique, complementary to BST, that was rigorously quantitative for protein *S*-nitrosation, which did not introduce disulfide bonds, the latter being a hallmark of the BST methodologies, including SNO-RAC. The need to avoid the introduction of disulfides rests upon the desire to extend d-Switch to parallel quantitation of endogenous disulfides. In contrast to BST and SNO-RAC methods, d-Switch carries through both labeled and unlabeled proteins, which is of course essential to quantitate nitrosated *versus* free thiol for any cysteine residue (Figure 1). Moreover in SNO-RAC, parallel samples are mixed and quantitated using iTRAQ reagents, a relative quantitation approach that reduces precision; d-Switch uses absolute quantitation obtained from single sample analysis.

SNOSID, SNO-RAC, and other BST modifications employ avidin or similar affinity columns or beads to concentrate the labeled peptides that were *S*-nitrosated in the test sample (14, 57–59). Although peptide loss occurs through inefficient digestion and retention on solid supports, affinity concentration has the potential to identify low abundance proteins, whereas d-Switch relies on the limit of detection of the mass spectrometer. If nitrosated proteins can be quantified at low nano- to picomolar levels with modern MS instruments, nitrosated proteins can be unambiguously identified by comparing the nondeuterated tandem mass spectra to the deuterated spectra. d-Switch peptides are readily identified and quantified by adapting currently available data-

base search methods used in established methods such as SILAC (60, 61).

To test the potential of d-Switch, the gel band excised for GST-P1 analysis from incubation of SH-SY5Y cells with CysNO (2 mM) was further interrogated using Mass Matrix, yielding approximately 50 proteins with acceptable coverage. Twenty peptides derived from these proteins showed NEM modification only, representing non-nitrosated cysteine residues (Supplementary Table S1); 8 were unambiguously identified by d-Switch to be nitrosated after treatment with CysNO (Table 1), 3 of which showed <10% nitrosation. Of the remaining 5 proteins, ubiquitin carboxyl-terminal esterase L1 (UCHL1) and PARK7 protein DJ-1, both nitrosated at two cysteine residues, deserve comment (Figure 5, panels B and C). UCHL1 has been identified as a major target of oxidative stress contributing to Parkinson’s and Alzheimer’s diseases, at least in part by oxidation of cysteine residues, but nitrosation has not been previously noted (62–64). DJ-1 has also been implicated in mediating oxidative stress in Parkinson’s disease, and furthermore nitrosation of key cysteine residues has been reported (65). A combination of cysteine mutants and BST was not able to differentiate between Cys46 and Cys53 nitrosation; however, d-Switch quantitatively revealed the degree of nitrosation: 29% and 56%, respectively. The generalization of d-Switch to quantitation of nitrosated and non-nitrosated cysteine residues in cell culture makes this method a valuable complement to published BST methods. It should also be emphasized that in the absence of exogenous NO-donor, the proteins identified in Table 1 and Supplementary Table S1 were identified and observed not to be nitrosated.

In summary, the simple d-Switch proteomic technique is introduced, using commercially available thiol-alkylating isotopologues, which identifies and quantitates protein targets of *S*-nitrosation by measuring the relative amounts of each peptide-SNO *versus* peptide-SH. The method was used to measure GST-P1 nitrosation induced by 5 classes of NO-donors that were seen to be differentiated in extent of nitrosation and sensitivity to cysteine and oxygen. The methodology was further extended to neuronal cell culture, wherein proteomic analysis identified protein thiols that were either sensitive or insensitive to nitrosation by exogenous nitrosothi-

TABLE 1. Nitrosated proteins detected by d-Switch analysis of ~20 kDa gel band from cell lysates after incubation of SH-SY5Y neuroblastoma cells with CysNO

	Av. mass	Sequence coverage (%)	Peptides	Nitrosation (%)
Glutathione-S-transferase P1-1 (GST-P1)	23724.66	69	ASC ₄₇ LYGQLPK	50.5
Cofilin 1(CFL1)	18871.37	59	AVLFC ₃₉ LSEDKK	37.5
Ubiquitin carboxyl-terminal esterase L1 (UCHL1)	25443.28	53	QTIGNSC ₉₀ GTIGLIHAVANNQDK NEAIQAAHDAVAQEGQC ₁₅₂ R	66.6 48.0
PARK7 Protein DJ-1	20134.87	64	VTVAGLAGKDPVQC ₄₆ SR DVVIC ₅₃ PDASLEDAKK	28.6 56.2
Chromobox protein homolog 3 (CBX3)	21055.23	38	GFTDADNTWEPEENLDC ₆₉ PELIEAFLNSQ	16.6
Phosphatidylethanolamine binding protein 1 (PEB1)	21175.52	72	APVAGTC ₁₆₈ YQAEWDDYVPK	<10
Transgelin-2 (TAGLN2)	21329.69	58	QYDADLEQILIQWITTQC ₃₈ R DGTVLC ₆₃ ELINALYPEGQAPVKK	<10
Ribosomal protein S5 (RPS5)	23120.19	43	TIAEC ₁₇₂ LADELINAAK	<10

ol; the in-sample quantitation of both nitrosated and non-nitrosated cysteines providing an advantage over other BST methods. Since all NO-donors induced significant

thiol oxidation, parallel or consequent to S-nitrosation, the extension of the d-Switch method to allow quantitation of protein disulfide is important and is in progress.

METHODS

Materials. All chemicals and reagents were purchased from Sigma (St Louis, MO) or Aldrich (Milwaukee, WI) unless otherwise mentioned. *d*₅-NEM was purchased from Cambridge Isotopes (Andover, MA). CysNO (66) and GTN (67) were synthesized as described in the literature. CysNO, GSNO, and DEA/NO were freshly prepared just before experiment and concentrations were determined spectrophotometrically prior to experiment according to the literature (68). Synthesis of ODZ1 is described in Supporting Information.

GST-P1 Expression and Purification. Standard recombinant DNA techniques were followed as described by Sambrook *et al.* (69) and detailed in the Supporting Information, as are detailed methods for NO treatment of intact *E. coli* cells and d-Switch analysis elemental information.

Biotin-Switch (BST) Technique. The Jaffrey *et al.* BST technique was followed with some modifications (13) as described in Supporting Information. The reaction buffer used in BST and GST-P1 oxidation experiments was HEPES (pH 7.4, 250 mM), EDTA (1 mM) and neocuproine (0.1 mM).

Analysis of GST-P1 Oxidation. For analysis of oxidation induced by NO-donors, GST-P1 was incubated with desired concentration of NO-donors at 37 °C for 30 min. SDS-PAGE was performed under nonreducing conditions followed by Coomassie blue staining.

d-Switch Method for Quantitation of Nitrosation. All steps were performed in the dark in amber colored vials. Purified GST-P1 protein storage buffer was exchanged with reaction buffer containing 40 mM ammonium bicarbonate, 1 mM EDTA, and 0.1 mM neocuproine at pH 7.4. Additional use of DTPA in the reaction buffer did not perturb the degree of nitrosation observed on reaction of GST-P1 with CysNO (Supporting Information). After incubation with NO-donor for 30 min, the unreacted thiols

were blocked with 20 mM NEM and 5% SDS by alternating between sonicating and shaking in a 55 °C water bath for 30 min. The unreacted NEM was removed with a Microcon YM-10 filter device, and the protein was eluted with 40 mM ammonium bicarbonate. For the labeling step, a saturated solution of CuCl (100 μM) was prepared as described (38). The labeling reaction was carried out at 25 °C for 60 min with 5 mM sodium ascorbate, 1 μM CuCl, and 2 mM *d*₅-NEM. The fresh samples were then run on SDS-PAGE and subjected to trypsinolysis using pierce in-gel digestion kit (cat. no. 89871). The samples were then analyzed using an Agilent 1100 series LC coupled to an Agilent 6310 ESI Ion Trap mass spectrometer in positive ion mode. The precision of the analysis is high for replicate experiments on the same batch of recombinant protein as reported.

Cell Culture and Extract Preparation for MS Analysis. SH-SY5Y cells were treated with CysNO for 20 min. After the treatment, cells were washed, lysed, and centrifuged. Precipitated protein was collected, washed, treated with labeling solution (1 mM biotin HPDP or 2 mM *d*₅-NEM, 5 mM sodium ascorbate, 1 μM CuCl), and incubated at 25 °C for 60 min. Samples were run on SDS gel, and the GST-P1 containing region was excised and subjected to in-gel digestion.

Mass Spectroscopic Analysis of Tryptic Digests. The tryptic digest samples were analyzed by using a hybrid linear ion trap FT-ICR mass spectrometer (LTQ-FT-ICR, Thermo Electron Corp., Bremen, Germany) equipped with a nanospray source, and nano-HPLC with autosampler (Dionex, Sunnyvale, CA). Details for MS analysis and database searching are provided in Supporting Information.

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Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

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